PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{6} : **C07K 1/00, G01N 1/00**	**A1**	(11) International Publication Number: **WO 99/46282** (43) International Publication Date: 16 September 1999 (16.09.99)

(21) International Application Number: PCT/US99/05155

(22) International Filing Date: 10 March 1999 (10.03.99)

(30) Priority Data:
60/077,889   13 March 1998 (13.03.98)   US

(63) Related by Continuation (CON) or Continuation-in-Part
(CIP) to Earlier Application
US   60/077,889 (CON)
Filed on   13 March 1998 (13.03.98)

(71) Applicant *(for all designated States except US)*: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WEISSBACH, Lawrence [–/US]; Boston, MA (US). LEWIS, Valerae [–/US]; Newton, MA (US). O'REILLY, Michael [–/US]; Newton, MA (US).

(74) Agent: CREASON, Gary, L.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110–2804 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

---

(54) Title: PLASMINOGEN–RELATED GENE B POLYPEPTIDES

(57) Abstract

A method of inhibiting angiogenesis or neoplastic growth, or both, in a bird or mammal is disclosed. The method involves identifying an animal that has, or is at risk for, unwanted angiogenesis or neoplastic growth, or both, and administering to the animal an amount of a plasminogen–related gene–B polypeptide sufficient to inhibit the angiogenesis or neoplastic growth.

- 1 -

PLASMINOGEN-RELATED GENE B POLYPEPTIDES

Field of the Invention

5   The invention relates to cell biology,
angiogenesis, and oncology.

Background of the Invention

       Plasminogen, a serine proteinase precursor
secreted by the liver, plays a role in dissolving blood
clots.   The active two-chain proteinase plasmin is
15 produced from plasminogen by cleavage of a specific Arg-
Val peptide bond by tissue-type or urokinase-type
plasminogen activator along with autocatalytic removal of
an N-terminal preactivation peptide.   Plasmin attacks
fibrin, a principal component of clots, thereby
20 facilitating clot lysis.   In addition to its well-
characterized thrombolytic capability, plasmin, along
with other proteinases, has been implicated as a mediator
of tissue remodeling during development, cartilage
destruction in osteoarthritis, and basement membrane
25 invasion by metastatic tumor cells.
       A plasminogen-related gene B polypeptide (PRG-B
polypeptide; also known as plasmilar or plasminogen-
related protein [PRP]) has been described by Weissbach,
U.S. Patent No. 5,545,717; Weissbach et al., *Biochem.*
30 *Biophys. Res. Commun.* **186**:1108 (1992); and Ichinose et
al., *Biochemistry* 31:3113-3118 (1992).   After cleavage of
the signal peptide, the mature PRG-B polypeptide is 77

- 2 -

amino acids in length and is highly homologous to the N-terminal preactivation peptide of plasminogen.

Summary of the Invention

It has been discovered that a PRG-B polypeptide
5 inhibits angiogenesis in an *in vitro* avian model, and also inhibits neoplastic growth in an *in vivo* mammalian model.

Accordingly, the invention features a method of inhibiting angiogenesis in a bird or mammal, e.g, a
10 human. The method involves identifying a bird or mammal that has, or is at risk for, unwanted angiogenesis; and administering to the animal an amount of the PRG-B polypeptide sufficient to inhibit angiogenesis. Conditions involving unwanted angiogenesis include non-
15 cancerous growths, arthritis, and diabetic retinopathy.

The invention also features a method for decreasing or preventing a neoplastic growth such as a cancer of the lung, breast, or prostate gland. The method involves identifying an animal that has, or is at
20 risk for, a neoplastic growth; and administering to the animal an amount of PRG-B polypeptide sufficient to decrease or prevent the neoplastic growth.

The invention further includes (1) a pharmaceutical composition for treating a condition
25 associated with unwanted angiogenesis or neoplastic growth in a subject, the composition including a PRG-B polypeptide; (2) the use of a PRG-B polypeptide for preparation of the pharmaceutical composition; and (3) the use of an expression vector encoding a PRG-B
30 polypeptide for preparation of the pharmaceutical composition.

The PRG-B polypeptide used in the methods of this invention can be full length, mature plasmilar, whose amino acid sequence is disclosed by Weissbach et al.,

- 3 -

Biochem. Biophys. Res. Commun. **186**:1108 (1992) (GenBank
Accession No. M93143). Alternatively, the PRG-B
polypeptide can be the functional equivalent of
plasmilar, i.e., a peptide mimetic based on plasmilar, or
5 a polypeptide that: (1) displays antiangiogenic or
anticancer activity, and (2) includes an amino acid
sequence sharing at least 70% identity with the full
length, mature plasmilar amino acid sequence.
Preferably, sequence identity is at least 80%, and more
10 preferably, at least 90%.

          A specific example of a PRG-B polypeptide useful
in the methods of this invention consists of the
following amino acid sequence:

          MRGSHHHHHH TDPHASSVPR VDPLDDYVNT
15   QGPSLFSVTK KQLGAGSREE CAAKCEEDKE
          FTCRAFQYHS KEQQCVIMAE NRKSSIIIRM RDAVLFEK
          (SEQ ID NO:4).

          In this particular sequence (SEQ ID NO:4), the
amino-terminal glutamate residue (E) of full-length,
20 mature plasmilar (position 22) is replaced with an
aspartate residue (D). In this sequence (SEQ ID NO:4),
the six contiguous histidine residues (positions 5-10)
function as a "polyhistidine tag" useful in purification
of the recombinant polypeptide (by means of its
25 interaction with nickel-NTA agarose). The remaining
amino acid residues between the amino terminal methionine
of the polypeptide and the amino terminus of the full-
length, mature plasmilar are artifacts of DNA cloning
procedures used to product the recombinant PRG-B
30 polypeptide.

          As used herein, "sequence identity" means the
percentage of identical subunits at corresponding
positions in two sequences when the two sequences are
aligned to maximize subunit matching, i.e., taking into
35 account gaps and insertions. For example, if 7 positions

- 4 -

in a sequence 10 amino acids are identical to the
corresponding positions in a second 10-amino acid
sequence, the two sequences have 70% sequence identity.
Sequence identity is typically measured using sequence
5   analysis software such as the Sequence Analysis Software
Package of the Genetics Computer Group, University of
Wisconsin Biotechnology Center, 1710 University Avenue,
Madison, WI 53705, or BLAST programs, provided as a
service by the National Center for Biotechnology
10   Information, are useful for making sequence comparisons.
The programs are described in detail by Karlin et al.,
Proc Natl Acad Sci USA 87:2264 (1990) and 90:5873 (1993),
and Altschul et al., *Nucl Acids Res* 25:3389 (1997), and
are available on the Internet at
15   http://www.ncbi.nlm.nih.gov.

Another preferred, non-limiting example of a
mathematical algorithm utilized for the comparison of
sequences is the algorithm of Myers and Miller, CABIOS
(1989). Such an algorithm is incorporated into the ALIGN
20   program (version 2.0) which is part of the GCG sequence
alignment software package. When utilizing the ALIGN
program for comparing amino acid sequences, a PAM120
weight residue table, a gap length penalty of 12, and a
gap penalty of 4 can be used.

25   The percent identity between two sequences can be
determined using techniques similar to those described
above, with or without allowing gaps. In calculating
percent identity, only exact matches are counted.

In the case of polypeptide sequences that are less
30   than 100% identical to a reference sequence, the non-
identical positions are preferably, but not necessarily,
conservative substitutions for the reference sequence.
Conservative substitutions typically include
substitutions within the following groups: glycine and
35   alanine; valine, isoleucine, and leucine; aspartic acid

- 5 -

and glutamic acid; asparagine and glutamine; serine and
threonine; lysine and arginine; and phenylalanine and
tyrosine. For example, a 10 amino acid polypeptide is
said to be at least 80% conserved if it differs from a
5 reference polypeptide by no more than two non-
conservative substitutions.

PRG-B polypeptides can be administered directly.
Direct administration of PRG-B polypeptides can be
systemic, for example, intravenous. Alternatively, PRG-B
10 polypeptides can be administered locally, for example, by
direct injection into a tumor. Direct administration
also can be from an implant, which provides continuous,
slow release of the PRG-B polypeptide.

In addition, PRG-B polypeptides can be
15 administered indirectly by means of an expression vector.
An expression vector is any nucleic acid molecule or
virus containing regulatory elements or reporter genes
for the purpose of expression of a given gene in
prokaryotic or eukaryotic cells or organisms. Such
20 vectors can be introduced into a cell by means of
molecular biological techniques. After introduction into
the cell, this nucleic acid can exist extrachromosomally
or become integrated into the host genome. Such cells
can also be administered to the animal.

25   One or more additional compounds, including other
angiogenesis inhibitors or antimitotic drugs, can be
administered before, concurrently, or after
administration of a PRG-B polypeptide. When these
compounds are administered concurrently, they can be
30 present in one single pharmaceutical composition.

Other features and advantages of the invention
will be apparent from the following detailed description,
and from the claims.

- 6 -

Brief Description of the Drawings

Fig. 1 is a graph of mice tumor volume versus days of treatment with saline or PRG-B polypeptide.

Detailed Description

5   The presence of blood vessels in non-cartilaginous tissues of birds or mammals is maintained by a delicate balance of inhibitors and stimulators of angiogenesis. Changes in this balance can promote or inhibit development of blood vessels. The discovery that a PRG-B

10 polypeptide plays a role in the regulation of angiogenesis, and thus tumor growth, has led to the methods of this invention.

       Amounts of a PRG-B polypeptide suitable for use in this invention can be produced by conventional

15 recombinant methods employing cultured host cells. For example, recombinant PRG-B polypeptides (rPRG-B) can be produced as described in Example 1 below. The host cells can be prokaryotes, e.g., *E. coli*. Alternatively, the host cells can be eukaryotes, e.g., yeast, insect cells,

20 or mammalian cells. Nucleic acid vectors containing a rPRG-B coding region operably linked to suitable expression control sequences can be introduced into the host cells by viral infection, receptor-mediated endocytosis, liposome fusion, or any other standard

25 technique. Extraction and purification of rPRG-B expressed by cultured host cells can be carried out using techniques known in the art, including, for example, the affinity purification procedure described in Example 1 below.

30   Administration of PRG-B polypeptides according to the invention can be carried out according to various standard methods, including intravenous, subcutaneous, intra arterial, intraperitoneal, transmucosal, oral, and intrapulmonary administration. In addition, an implant

- 7 -

that allows slow release can be used to administer the
PRG-B polypeptides to the patient. The PRG-B
polypeptides can be administered in combination with one
or more additional active agents, e.g., a
5 chemotherapeutic drug such as taxol.

PRG-B polypeptides can be administered in dosages
comparable to dosages of other therapeutically
administered proteins. Typically, dosage levels will be
designed to produce a serum concentration of about 1-100
10 ng/ml when administered systemically. Alternatively,
PRG-B polypeptides can be injected directly into a target
tissue such as a tumor. This may result in local
concentrations of PRG-B polypeptides above 100 ng/ml
while systemic serum concentrations remain below 1 ng/ml.
15 Local delivery techniques are further discussed below.
Optimal dosage for a given patient depends on factors
such as the patient's weight, age, gender, and treatment
indication, and can be determined by one of ordinary
skill in the art.

20   The data obtained from cell culture assays and
animal studies can be used in formulating a range of PRG-
B polypeptide dosage for use in humans. For example, a
titration curve can be determined by repeating the
procedures described in Example 1 below with varying
25 concentrations of rPRG-B. The dosage lies preferably
within a range of circulating concentrations that include
the ED_{50} with little or no toxicity. The dosage may vary
within this range depending upon the dosage form employed
and the route of administration utilized. Levels of PRG-
30 B polypeptides in plasma can be measured by conventional
analytical methods, e.g., high performance liquid
chromatography or radioimmunoassay.

Additional guidance on methods of determining
dosages can be found in standard references, for example,
35 Spilker, *Guide to Clinical Studies and Developing*

- 8 -

Protocols, Raven Press Books, Ltd., New York, 1984, pp.
7-13 and 54-60; Spilker, *Guide to Clinical Trials*, Raven
Press, Ltd., New York, 1991, pp. 93-101; Craig et al.,
Modern Pharmacology, 2d ed., Little Brown and Co.,
5 Boston, 1986, pp. 127-133; Speight, *Avery's Drug
Treatment: Principles and Practice of Clinical
Pharmacology and Therapeutics*, 3d ed., Williams and
Wilkins, Baltimore, 1987, pp. 50-56; Tallarida et al.,
Principles in General Pharmacology, Springer-Verlag, New
10 York, 1998, pp. 18-20; and Olson, *Clinical Pharmacology
Made Ridiculously Simple*, MedMaster, Inc., Miami, 1993,
pp. 1-5.

Pharmaceutical compositions containing a PRG-B
polypeptide for use in the methods of the present
15 invention can be formulated in conventional manner using
one or more physiologically acceptable carriers or
excipients.

PRG-B polypeptides may be formulated for
administration by inhalation or insufflation (either
20 through the mouth or the nose) or oral, buccal,
pulmonary, nasal, parenteral, or rectal administration.

For oral administration, the pharmaceutical
compositions may take the form of, for example, tablets
or capsules prepared by conventional means with
25 pharmaceutically acceptable excipients such as binding
agents (for example, pregelatinised maize starch,
polyvinylpyrrolidone or hydroxypropyl methylcellulose);
fillers (for example, lactose, microcrystalline cellulose
or calcium hydrogen phosphate); lubricants (for example,
30 magnesium stearate, talc or silica); disintegrants (for
example, potato starch or sodium starch glycolate); or
wetting agents (for example, sodium lauryl sulphate).
The tablets may be coated by methods well known in the
art. Liquid preparations for oral administration may
35 take the form of, for example, solutions, syrups or

- 9 -

suspensions, or they may be presented as a dry product
for constitution with water or other suitable vehicle
before use. Such liquid preparations may be prepared by
conventional means with pharmaceutically acceptable

5   additives such as suspending agents (for example,
sorbitol syrup, cellulose derivatives or hydrogenated
edible fats); emulsifying agents (for example, lecithin
or acacia); non-aqueous vehicles (for example, almond
oil, oily esters, ethyl alcohol or fractionated vegetable

10 oils); and preservatives (for example, methyl or propyl-
p-hydroxybenzoates or sorbic acid). The preparations may
also contain buffer salts, flavoring, coloring and
sweetening agents as appropriate.   Preparations for
oral administration may be suitably formulated to give

15 controlled release of the active compound. To avoid
degradation of PRG-B polypeptides in the gastrointestinal
tract, delivery systems such as bacterial toxin fusion
proteins or liposomes can be used. Such delivery systems
are described in Mestecky et al., *Behring Inst. Mitt.*

20 98:33-43 (1997); Storm et al., *Hybridoma* 16:119-125
(1997); and Rowlinson-Busza et al., *Curr. Opin. Oncol.*
4:1142-1148 (1992).

    For buccal administration the compositions may
take the form of tablets or lozenges formulated in

25 conventional manner.

    For administration by inhalation, the PRG-B
polypeptides used according to the methods of the present
invention is conveniently delivered in the form of an
aerosol spray from pressurized packs or a nebulizer, with

30 the use of a suitable propellant. In the case of a
pressurized aerosol the dosage unit may be determined by
providing a valve to deliver a metered amount. Capsules
and cartridges of, for example, gelatin for use in an
inhaler or insufflator may be formulated containing a

- 10 -

powder mix of the compound and a suitable powder base
such as lactose or starch.

The PRG-B polypeptides can be formulated for
parenteral administration by injection, for example, by
5   bolus injection or continuous infusion. Formulations for
injection may be presented in unit dosage form, for
example, in ampules or in multi-dose containers, with an
added preservative. The compositions may take such forms
as suspensions, solutions or emulsions in oily or aqueous
10 vehicles, and may contain formulatory agents such as
suspending, stabilizing and/or dispersing agents.
Alternatively, the active ingredient may be in powder
form for constitution with a suitable vehicle, for
example, sterile pyrogen-free water, before use.
15   PRG-B polypeptides can also be formulated in
rectal compositions such as suppositories or retention
enemas, for example, containing conventional suppository
bases such as cocoa butter or other glycerides.

In addition to the formulations described
20 previously, PRG-B polypeptides may also be formulated as
a depot preparation (e.g., an implant). Such long acting
formulations may be administered by implantation (for
example subcutaneously or intramuscularly) or by
intramuscular injection. Thus, for example, PRG-B
25 polypeptides can be formulated with suitable polymeric or
hydrophobic materials (for example as an emulsion in an
acceptable oil) or ion exchange resins, or as sparingly
soluble derivatives, for example, as a sparingly soluble
salt.
30   Targeting of a PRG-B polypeptide to neoplastic
cells can be achieved by local injection of PRG-B
polypeptide compositions directly into tumors. For
enhanced targeting, the compositions can contain
components that specifically bind to neoplastic cells,
35 e.g., antibodies or polypeptides specific for cancer

- 11 -

antigens, preferably cell-surface cancer antigens.
Alternatively, PRG-B polypeptides can be targeted to
sites of abnormally high angiogenesis by including in the
composition polypeptides, e.g., antibodies or fragments
5   thereof, that bind to antigens associated with sites of
angiogenesis.

PRG-B polypeptides can also be introduced into a
patient by expressing within the cells of the patient a
nucleic acid construct containing expression control
10   sequences operably linked to a sequence encoding a PRG-B
polypeptide. The nucleic acid construct is derived from
a non-replicating linear or circular DNA or RNA vector,
or from an autonomously replicating plasmid or viral
vector; or the construct is integrated into the host
15   genome. Any vector that can transfect a mammalian cell
may be used in the methods of the invention. Methods for
constructing expression vectors are well known in the art
(see, e.g., *Molecular Cloning: A Laboratory Manual*,
Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd
20   Edition, Cold Spring Harbor, New York, 1989).

In these vectors, promoters are operably linked to
the nucleic acid sequence encoding PRG-B polypeptides.
Any promoter that can direct a high level of
transcription initiation in the target cells may be used
25   in the invention. Such target cells include cancer
cells, healthy cells surrounding cancer cells, and any
other cell type in close proximity to the area affected.
Non-tissue specific promoters, such as the
cytomegalovirus (DeBernardi et al., *Proc. Natl. Acad.*
30   *Sci. USA* 88:9257-9261, 1991, and references therein),
mouse metallothionine I gene (Hammer et al., *J. Mol.*
Appl. Gen. 1:273-288, 1982), HSV thymidine kinase
(McKnight, *Cell* 31:355-365, 1982), and SV40 early
(Benoist et al., *Nature* 290:304-310, 1981) promoters may
35   be used in methods of the invention, as expression of

- 12 -

PRG-B polypeptides in the methods of the invention would
not be expected to adversely affect transfected cells.
The above-described nucleic acid constructs and vectors
can be introduced into target cells by any standard
5 method: e.g., as naked DNA, or by liposome fusion,
biolistic transfer, electroporation, erythrocyte ghosts,
or microsphere methods (microparticles; see, e.g.,
U.S. Patent No. 4,789,734; U.S. Patent No. 4,925,673;
U.S. Patent No. 3,625,214; Gregoriadis, *Drug Carriers in*
10 *Biology and Medicine*, pp. 287-341, Academic Press, 1979).
    Alternatively, one can employ a viral-based vector
as a means for introducing the nucleic acid into the
cells of the animal. Preferred viral vectors include
those derived from replication-defective hepatitis
15 viruses (e.g., HBV and HCV), retroviruses (see, e.g.,
WO89/07136; Rosenberg et al., *N. Eng. J. Med.* **323**(9):570-
578, 1990), adenovirus (see, e.g., Morsey et al., *J.
Cell. Biochem.*, **Supp. 17E**, 1993), adeno-associated virus
(Kotin et al., *Proc. Natl. Acad. Sci. USA* **87**:2211-2215,
20 1990), replication defective herpes simplex viruses (HSV;
Lu et al., Abstract, page 66, *Abstracts of the Meeting on
Gene Therapy*, Sept. 22-26, 1992, Cold Spring Harbor
Laboratory, Cold Spring Harbor, New York), canary pox
virus, and any modified versions of these vectors.
25   Rather than introduction of the naked vector into
the patient, delivery of PRG-B polypeptides can be
accomplished by transfecting cells (e.g., primary cells
of the patient) *in vitro* with an expression vector
encoding PRG-B polypeptides; culturing the cells to
30 produce a stably transformed, uniform population; and
implanting the PRG-B polypeptide-secreting population of
cells into the patient.
    Whichever method is used to deliver PRG-B
polypeptides into a patient, response to treatment can be
35 measured by various suitable methods known in the art.

- 13 -

For example, a solid tumor can be imaged using magnetic resonance imaging techniques after PRG-B polypeptide administration to determine efficacy. Alternatively, a surrogate marker for tumor growth, such as carcino-
5 embryonic antigen or prostate-specific antigen, can be measured in patient samples such as a blood sample. In non-cancerous conditions, angiogenesis can be monitored before and after PRG-B polypeptide administration by direct examination of the affected tissue or using dyes
10 or radioactive tracers to image blood flow. Possible adverse effects of PRG-B polypeptide administration can include inhibition of wound healing, which should be monitored as well.

Contraindications for administration of a PRG-B
15 polypeptide include situations in which inhibiting angiogenesis is especially detrimental to the patient. For example, caution should be exercised when administering a PRG-B polypeptide to infants, pregnant women, or patients experiencing difficulty in wound
20 healing.

One skilled in the art can, based on the above disclosure and the examples described below, utilize the present invention to its fullest extent. The following examples are to be construed as merely illustrative of
25 how one skilled in the art can make and use PRG-B polypeptides and are not limitative of the remainder of the disclosure in any way. Any publications cited in this disclosure are hereby incorporated by reference.

Example 1: Production of
30 Recombinant PRG-B Polypeptides

A recombinant fusion protein consisting of the PRG-B polypeptide fused to a hexahistidine-containing peptide at the N-terminus was constructed (SEQ ID NO:4). The hexahistidine feature allowed purification of the

- 14 -

recombinantly produced polypeptide by means of a nickel-NTA resin (Qiagen).

PCR was performed using the full-length PRG-B cDNA (as described by Weissbach et al.; GenBank Accession No.
5 M93143) as a template and two oligonucleotide primers harboring internal restriction sites, that facilitated subcloning into the pQE-31 plasmid vector (Qiagen). The pQE vectors expressed inserted genes under the control of the *E. coli* phage T5 promoter and the *lac* operator. The
10 sense primer was 5'-ACTTCACCCGGGCAAGTCGACCCTCTGGATGAC-3' (SEQ ID NO:1), corresponding to nucleotides 107-139 of the cDNA; and the antisense primer was 5'-TTCGGATCCCAGTCTAGAACTCTGAAAG-3' (SEQ ID NO:2), corresponding to nucleotides 365-392 of the cDNA. The
15 PCR was performed using a BIOSCYCLER (IBI) with the following cycle parameters: 94°C for 90 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. A total of 30 cycles were performed. The 286 bp PCR product and the pQE-31 plasmid were digested with BamHI and SalI
20 restriction enzymes, passed over a Chromaspin-100 pre-packed spin filtration column (Clontech) to remove the small DNA fragments released by the enzyme digestions, and ligated together using T4 DNA ligase.

Ligated DNA was transformed into M15 cells
25 containing the pREP4 plasmid (Qiagen), which expresses high levels of the *lac* repressor, ensuring tight control over transcription of inserted genes. Transformants were analyzed by BamHI/SalI digestion, and insert-containing plasmid clones were sequenced using a primer available
30 from Qiagen (5'-CGGATAACAATTTCACACAG-3') (SEQ ID NO:3), which lies 27 bp upstream from the initiating methionine codon. One DNA isolate containing the correct sequence in frame with the histidine tag was used for large scale purification of the PRG-B recombinant fusion protein.

- 15 -

The DNA sequence predicted a fusion protein of 11,221 daltons and an isoelectric point of 7.54.

Purification of the fusion protein under denaturing conditions was accomplished as follows: Ten
5   milliliters of an overnight culture of the M15 cells harboring the recombinant plasmid were added to 500 ml of LB media containing ampicillin and kanamycin, and shaken at 37°C until the OD_{600} was 0.8-0.9. IPTG was added to a final concentration of one millimolar, and the culture
10 was shaken for an additional three to four hours.

The cells were harvested by centrifugation, and the cell pellet was dissolved in 5 ml of Buffer A (8 M urea, 0.1 M NaH_2PO_4, 0.01 M imidazole, 10 mM ß-mercaptoethanol, and 0.01 M Tris at pH 8.0) per gram wet
15 weight. The solution was stirred for one hour at room temperature, and then centrifuged at 20,000 x g for 15 minutes. Three milliliters of a 50% slurry of Ni-NTA resin, previously equilibrated in Buffer A, were added to the supernatant, and the solution was again stirred at
20 room temperature for one hour. The mixture containing the resin and the bacterial extract was loaded into an empty column (14 cm x 3.5 cm) and washed sequentially with 50 ml of Buffer A and 10 column volumes of Buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M imidazole, 10 mM ß-
25 mercaptoethanol, and 0.01 M Tris at pH 6.25). Material bound to the resin was eluted with six column volumes of Buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.25 M imidazole, 10 mM ß-mercaptoethanol, and 0.01 M Tris at pH 6.25). Three milliliter fractions were collected, and ten milliliters
30 of each fraction was analyzed by sodium dodecylsulfate electrophoresis (SDS-PAGE) for the presence of the PRG-B fusion protein. The purified recombinant PRG-B polypeptide (rPRG-B) was dialyzed against PBS before its use in additional examples below.

- 16 -

Example 2:   rPRG-B Inhibits the
Growth of Malignant Tumors

          Syngeneic C57B16/J mice were implanted with Lewis
lung carcinomas as described in O'Reilly et al., *Cell*
5   79:315-328 (1994).   A suspension of 1 X 10^6 tumor cells in
100 microliters of PBS was injected into the subcutaneous
dorsa of the mice.   Tumors were measured with a dial-
caliper, and volumes were determined using the formula:
width^2 X length X 0.52.   This formula corresponds to the
10 general formula for the volume of an ellipsoid sphere,
where 0.52 approximates $\pi/6$, and is a standard
calculation for estimating tumor volumes.

          When the tumor volume reached about 160 mm^3, the
mice were randomized into two groups.   One group (n = 3)
15 received daily intraperitoneal injections of rPRG-B
suspended in PBS (as described in Example 1 above) at the
dose of 35 mg/kg body weight.   The control group (n = 3)
received PBS.   As shown in Fig. 1, the rPRG-B treatment
led to substantial decreases of average tumor size during
20 the observation period.   After 11 days of rPRG-B
treatment, the average tumor size was less than 1500 mm^3.
In contrast, the average tumor size was greater than 4500
mm^3 when the mice were treated with saline only.   The
error bars in the graph represent the 95% confidence
25 interval for each data point.   No toxicity or weight loss
was observed in any of the treated mice.

Example 3:   rPRG-B Inhibits Angiogenesis

          Angiogenesis inhibiting activity of rPRG-B was
examined using a standard chick chorioallantoic membrane
30 assay as described in Folkman, Angiogenesis and Its
Inhibitors, In: Important Advances in Oncology, De Vila
et al. editors, B. Lippincott Co., Philadelphia, pages
42-62 (1985).

- 17 -

Three-day old fertilized white Leghorn eggs
(Spafas, Norwich, CT) were cracked, and the embryos with
intact yolks were placed in petri-dishes. The embryos
were incubated for three days at 37°C and 3% carbon
5 dioxide. After incubation, a methlycellulose disc
containing 200 micrograms rPRG-B or nothing was placed
onto the chorioallantoic membrane of each embryo. After
48 hours, the membranes were examined under a
stereomicroscope for the presence or absence of new blood
10 vessels in the tissue under and around the disc. No
blood vessels were observed in the tissue under or around
the disk at the dosage of 200 micrograms for at least
three days after administering rPRG-B. In contrast, the
tissue under and round the control discs were heavily
15 infiltrated with new blood vessels. No toxicity was
observed in any of the chick embryos.

- 18 -

Claims

1.   A pharmaceutical composition for treating a
condition associated with unwanted angiogenesis or
neoplastic growth in a subject, the composition
5 comprising a PRG-B polypeptide.

2.   The use of a PRG-B polypeptide for preparation
of a pharmaceutical composition for treating a condition
associated with unwanted angiogenesis or neoplastic
growth in a subject.

10   3.   The use of an expression vector encoding a
PRG-B polypeptide for preparation of a pharmaceutical
composition for treating a condition associated with
unwanted angiogenesis or neoplastic growth in a subject.

4.   The composition of claim 1 or the use of claim
15 2 for direct administration to the subject.

5.   The composition or use of claim 4, wherein the
direct administration is by systemic, intravenous, local,
or implant means.

6.   The composition of claim 1 or the use of claim
20 2 for indirect administration to the subject.

7.   The composition of claim 1 or the use of claim
2, wherein the indirect administration is by means of an
expression vector encoding the PRG-B polypeptide.

8.   The composition or use of any preceding claim,
25 wherein the amount of the PRG-B polypeptide is sufficient
to inhibit the unwanted angiogenesis or neoplastic growth
in the subject.

- 19 -

9.   The composition or use of any preceding claim, wherein the condition treated is associated with lung cancer, breast cancer, or prostate cancer.

10.   The composition or use of any one of claims 5 1-9, wherein the condition treated is associated with a non-cancerous growth, arthritis, or diabetic retinopathy.

11.   The composition or use of any preceding claim, wherein the PRG-B polypeptide comprises the amino acid sequence of SEQ ID NO:4.

10   12.   A method of treating a subject having a condition associated with unwanted angiogenesis or neoplastic growth, the method comprising administering to the subject an amount of PRG-B polypeptide sufficient to inhibit the unwanted angiogenesis or neoplastic growth.

15   13.   The method of claim 12, wherein the PRG-B polypeptide is directly administered to the subject.

14.   The method of claim 13, wherein the direct administration is by systemic, intravenous, local, or implant means.

20   15.   The method of claim 12, wherein the PRG-B polypeptide is indirectly administered to the subject.

16.   The method of claim 15, wherein the indirect administration comprises introduction into the subject of an expression vector encoding the PRG-B polypeptide.

25   17.   The method of any one of claims 12-16, wherein the condition treated involves a neoplastic

- 20 -

growth associated with lung cancer, breast cancer, or
prostate cancer.

18.   The method of any one of claims 12-16,
wherein the condition treated involves unwanted
5 angiogenesis associated with a non-cancerous growth,
arthritis, or diabetic retinopathy.

19.   The method of any one of claims 12-16,
further comprising identifying a subject that has, or is
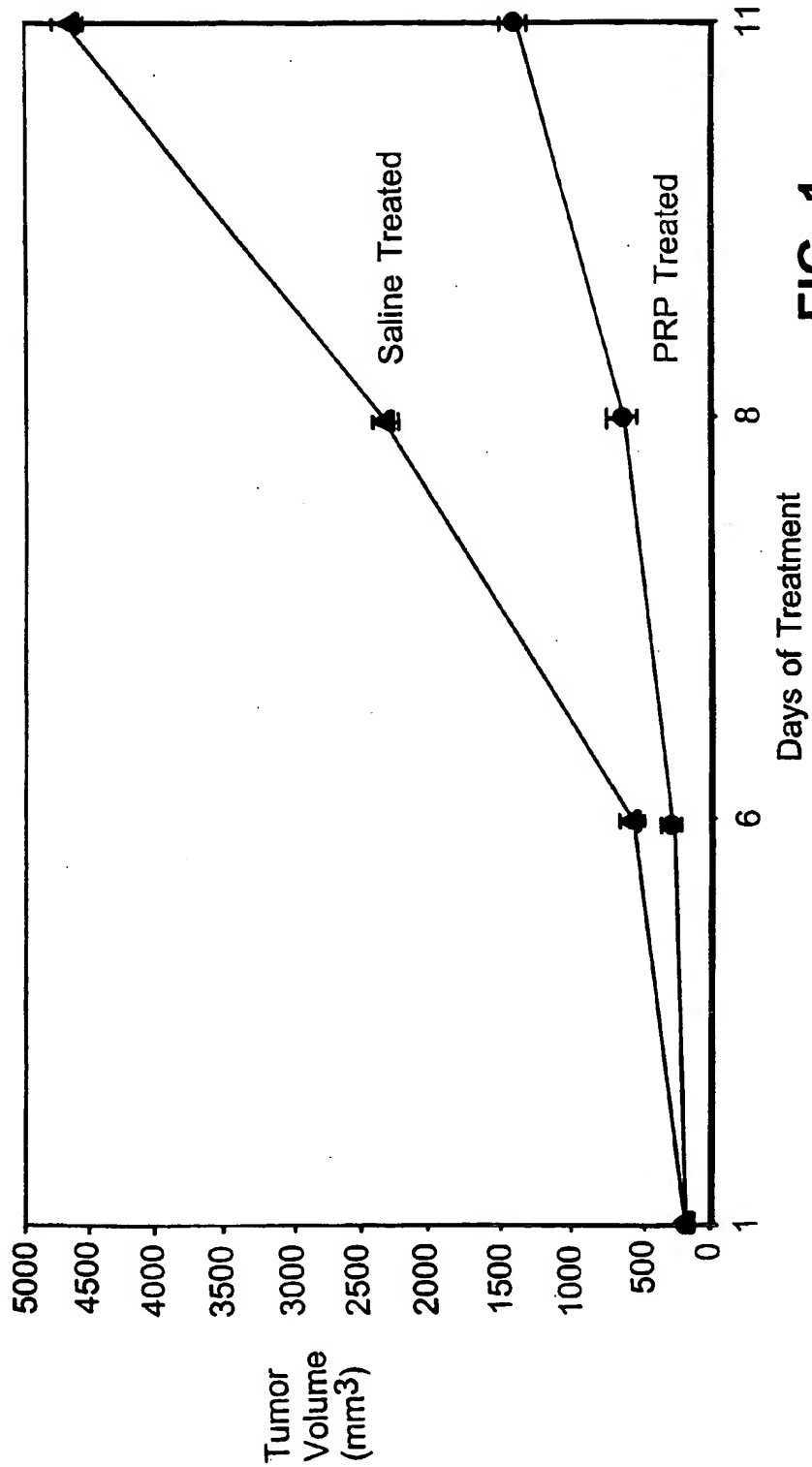at risk for, unwanted angiogenesis or neoplastic growth.

FIG. 1

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) . C07K 1/00; G01N 1/00
US CL : 530/350; 514·2

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 514·2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practicable. search terms used)

MEDLINE. APS
search terms: plasminogen-related gene B polypeptide. plasmilar. plasminogen-related protein

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate. of the relevant passages	Relevant to claim No.
X	US 5.545.717 A (WEISSBACH) 13 August 1996. see entire document. especially abstract and 2, lines 44-49.	1, 2, 8

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
20 APRIL 1999	**1 9 MAY 1999**
Name and mailing address of the ISA US Commissioner of Patents and Trademarks Box PCT Washington. D.C. 20231	Authorized officer SUSAN UNGAR
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT·ISA 210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No

PCT/US99-05155

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-11
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.
   1, 2 and 7

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT ISA 210 (continuation of first sheet (1)) (July 1992)

as plasmilar protein and that it has been described in US Patent No. 5,545,717 which reveals plasmilar protein. The recitation of a pharmaceutical composition in the preamble is viewed as an intended use and is given no weight in the examination of the claims. The claims read on the active ingredient per se, that is PRG-B polypeptide.

Therefore, the technical feature linking the inventions and the
species does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s), 1, 2 and 7 are drawn to a pharmaceutical composition comprising PRG-B polypeptide.
Group II, claim(s) 3, 4, 5 are drawn to a method of making a pharmaceutical composition comprising PRG-B polypeptide.
Group III, claim(s) 6 is drawn to a composition for indirect administration of PRG-B polypeptide to a subject.
Group IV, claims 12-19 are drawn to a method of treating a disease.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I is considered to be a pharmaceutical composition comprising PRG-B.
The special technical feature of Group II is considered to be a method of making a pharmaceutical composition comprising PRG-B polypeptide.
The special technical feature of Group III is considered to be a composition for indirect administration of PRG-B polypeptide.
The special technical feature of Group IV is considered to be a method of treating a disease.

Accordingly, Groups I-IV are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

This application contains claims in Group IV directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:
The generic claim is claim 12.

The claims are deemed to correspond to the species listed above in the following manner:

Species A - Claims 12, 13, 14, 17 drawn to a method comprising administering PRG-B polypeptide to a patient to treat a neoplastic condition.
Species B - Claims 12, 13, 14, 18 drawn to a method comprising administering PRG-B polypeptide to a patient to treat unwanted angiogenesis associated with a non-cancerous growth.
Species C - Claims 12, 13, 14, 19 drawn to a method comprising administering PRG-B polypeptide to identify a patient at risk for or who has unwanted angiogenesis or neoplastic growth.
Species D - Claims 12, 15, 16, 17 drawn to a method of administering an expression vector encoding PRG-B polypeptide to a patient to treat a neoplastic condition.
Species E - Claims 12, 15, 16, 18 drawn to a method of administering an expression vector encoding PRG-B polypeptide to a patient to treat unwanted angiogenesis associated with a non-cancerous growth.
Species F - Claims 12, 15, 16, 19 drawn to a method of administering an expression vector encoding PRG-B polypeptide to identify a patient at risk for or who has unwanted angiogenesis or neoplastic growth

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

Because they are drawn to methods which differ at least in objectives, method steps, reagents and/or dosages and/or schedules used, response variables and criteria for success.

The inventions listed as Groups I-IV and species A-F of Group IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT 13.2 they lack the same or corresponding special technical features for the following reasons.

The technical feature linking groups I-IV appears to be that they all relate to PRG-B polypeptide

However, the specification clearly admits on page 1 that the PRG-B polypeptide is known in the art and is known also